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Inflammatory and metabolic molecular markers in neuroendocrine tumors

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Epigraph

“ Não sou nada. Nunca serei nada. Não posso querer ser nada. À parte isso, tenho em mim todos os sonhos do mundo.”

Álvaro de Campos, Tabacaria

Resumo

Os tumores neuroendócrinos gastroenteropancreáticos (GEP-NETs) constituem um grupo heterogéneo e invulgar de neoplasias que sofreu um aumento exponencial da sua incidência e prevalência nas últimas décadas. Em paralelo com o aumento da taxa de GEP-NETs, obesidade, síndrome metabólica (SM) e diabetes também são dos principais problemas clínicos e de saúde pública em crescimento a nível mundial. A associação entre SM, os seus parâmetros e cancro foi recentemente mostradas para diferentes tipos de neoplasias, contudo a sua associação com os GEP-NETs ainda não foi reportada e merece ser explorada. O objetivo deste estudo foi avaliar a influência da SM e dos seus parâmetros individuais na expressão de diferentes marcadores moleculares que participam em vias metabólicas e inflamatórias em GEP-NETs e tecido peri-tumoral, bem como correlaciona-las com as características clinicas e patológicas do tumor. Com este objetivo, foram efetuadas análises imunohistoquímicas para avaliar a presença de IL-6, FOXM1 e IGF1R em cortes de GEP-NETs embebidos em parafina (n= 39) seguido por uma análise morfométrica para determinar a percentagem de área marcada no tumor (Ki-67, FOXM1 e IGF1R) e tecido peri-tumoral (IL-6). O presente estudo mostrou que a expressão de Ki-67, FOXM1 e IGF1R nos GEP-NETs não foi significativamente diferente na presença ou ausência de SM ou algum dos seus parâmetros. Porém, foi observada uma correlação positiva e significativa entre o Ki-67 e FOXM1 em NETs pancreáticos e ainda uma correlação positiva e significativa entre o FOXM1 e IGF1R em NETs gastrointestinais. A expressão do IL-6 no tecido peri-tumoral de NETs pancreáticos foi significativamente menor em doentes com obesidade central e em NETs gastrointestinais foi significativamente maior em doentes com HDL baixo. Não se observaram diferenças significativas de expressão do IL-6 na presença ou ausências de SM ou qualquer um dos seus outros parâmetros. Estes resultados sugerem que a presença da SM ou de algum dos seus parâmetros individuais não influencia significativamente nenhum dos marcadores estudados e assim alguma das vias com os quais estão relacionados nos GEP-NETs, exceto para o IL-6. Nos NETs gastrointestinais a expressão do IL-6 sugere que um baixo HDL pode contribuir para um microambiente inflamatório na área peri-tumoral. Por sua vez, o FOXM1 e a sua via parecem estar envolvidos na proliferação celular em GEP-NETs e a sua inibição poderá ser um alvo molecular importante para o tratamento destes tumores. Para além disso, os resultados sugerem que a expressão e atividade do FOXM1 em NETs gastrointestinais possa estar relacionada com a atividade do IGF1R. Estudos futuros serão necessários para perceber a associação e influência da SM e dos seus parâmetros individuais nos GEP-NETs e ainda para avaliar e compreender o potencial do FOXM1 e IGF1R como possíveis alvos no tratamento de GEP-NETs.

Abstract

Gastroenteropancreatic neuroendocrine tumors (GEP-NETs) comprise an uncommon and heterogeneous group of neoplasms which had an exponential increase in terms of incidence and prevalence throughout the last decades. In parallel with the increasing rate of GEP-NETs, obesity, metabolic syndrome (MS) and diabetes are also major and escalating public-health and clinical problems worldwide. The link between MS, its parameters and cancer has been recently demonstrated for several types of neoplasia, however the association with GEP-NETs has not so far been reported, although it deserves to be explored. The objective of this study was to evaluate the influence of MS criteria and their individual parameters in the expression of different molecular markers that participate in metabolic and inflammatory pathways in GEP-NETs and peri-tumoral tissue, as well as, their correlation with the tumor clinical and pathologic features. To achieve this aim, individual immunohistochemical studies for the presence of IL-6, FOXM1 and IGF1R were performed in sections of paraffin-embedded GEP-NETs tissue (n= 39) followed by an morphometric computerized analysis tool in order to assess the percentage of stained area in the both tumor (Ki-67, FOXM1 and IGF1R) and peri-tumoral (IL-6) area. The present study has shown that Ki-67, FOXM1 and IGF1R expression in pancreatic and gastrointestinal NETs was not significantly different between patients with or without MS or any of MS individual parameters. A significant positive correlation between the Ki-67 and FOXM1 expression in pancreatic and gastrointestinal NETs was observed, as well as, a significant positive correlation between the FOXM1 and IGF1R expression in gastrointestinal NETs. IL-6 expression at the peri-tumoral area in pancreatic NETs was significantly lower in patients with central obesity and significantly higher in gastrointestinal NETs of patients with low HDL. IL-6 expression in the peri-tumoral tissue was not found to be significantly different between patients with or without MS or any other of the reminder MS individual parameters in GEP-NETs. These results suggest that the presence of MS or any of its individual parameters does not significantly influences any of the studied markers and thus related pathways in GEP-NETs, except for IL-6. IL-6 expression at the peri-tumoral area of gastrointestinal NETs suggest that low HDL in gastrointestinal NETs may contribute to the inflammatory environment in the peri-tumoral area. FOXM1 and its pathway might be involved in GEP-NETs cell proliferation and thus FOXM1 inhibition could be an important molecular target for GEP-NETs treatment. Furthermore, the results suggest that FOXM1 expression could be stimulated and activated by IGF1R activity or vice-versa in gastrointestinal NETs. Future studies will be required in order to understand the link and influence of MS criteria, its individual parameters with GEP-NETs and to assess and understand the potential of FOXM1 and IGF1R as possible GEP-NETs treatment targets.

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Abbreviations List

BP- Blood pressure

CgA- Chromogranin A

ENETS- European Neuroendocrine Tumor Society

FOX- Forkhead box

FOXM1- Forkhead box M1

FOXO- Forkhead box O

FOXO3a- Forkhead box O3a

GI- Gastrointestinal

GEP-NETs- Gastroenteropancreatic neuroendocrine tumors

G1- Grade one

G2- Grade two

G3- Grade three

HDL- High-density lipoprotein

HPF- High power fields

IBD- Inflammatory Bowel Diseases

IGF1- Insulin-like growth factor one

IGF1R- Insulin-like growth factor one receptor

IL-1- Interleukin-1

IL-1β- Interleukin-1 beta

IL-6- Interleukin-6

IL-8- Interleukin-8

IL-10- Interleukin-10

IL-12- Interleukin-12

IL-17- Interleukin-17

IL-23- Interleukin-23

JAK-STAT- Janus kinase–signal transducer and activator of transcription

MANECs- Classified as mixed adenoneuroendocrine carcinomas

MAPK- Mitogen-activated protein kinase

MS- Metabolic syndrome

mTOR- Mammalian target of rapamycin

NE- Neuroendocrine

NETs- Neuroendocrine tumors

NF-kB- Nuclear factor-kappa B

PD-NEC- Poorly differentiated neuroendocrine carcinomas

PI3K- Phosphatidylinositol 3-kinase

PLC/PKC- Phospholipase C/Protein kinase C

ROS- Reactive oxygen species

STAT3- Signal transducers and activator of transcription 3

TGF-β- Transforming growth factor-beta

TKRs- Tyrosine kinase receptors

TNF-α- Tumor necrosis factor-alpha

T2D- Type 2 diabetes

VEGF- Vascular endothelial growth factor

WD-NETs- Well-differentiated neuroendocrine tumors

WHO- World Health Organization

Chapter 1- Introduction

1.1- Neuroendocrine Tumors

Neuroendocrine tumors (NETs) originate from neuroendocrine (NE) cells that can be found throughout the body and comprise a heterogeneous group of unusual neoplasms characterized by a common neuroectodermal embryological origin and different biological behavior (Pusceddu et al., 2015; Singh & Law, 2012; Xiaojiang Yi, 2013; Yao et al., 2008). NE cells, share features of neural and endocrine differentiation, belong to the diffuse endocrine system and are present in certain organs and glands, such as the thyroid, pancreas and adrenals, as well as, throughout the body, including the vascular system, respiratory tract and the gastrointestinal mucosa (Essand et al., 2011; Rindi & Wiedenmann, 2011; Uccella, Sessa, & Rosa, 2015). Regardless of their location, NETs share familiar biochemical features. These neoplastic cells present secretory granules, synaptic-like vesicles and assemble precursor molecules which are then processed and capable of producing hormones, peptides or amines (Essand et al., 2011; Rindi & Wiedenmann, 2011; Uccella, Sessa, & Rosa, 2015). Despite some resemblances, accordingly with their location in the human body these cells diverge in their tightly controlled ability to secrete and release hormones into the blood stream, adjacent cells or neurons and regulate various specific processes, such as, air flow, gastrointestinal secretion, blood pressure and motility (Andrew, Kramer, & Rawdon, 1998; Essand et al., 2011). NE differentiation can be found in many human neoplasms, such as colorectal and prostate cancer, and has been suggested as a marker of poor prognosis for a variety of carcinomas, having the ability to uptake/set free different NE substances (Parimi, Goyal, Poropatich, & J Yang, 2014; Rindi & Wiedenmann, 2011; Sun, 2004).

These uncommon and heterogeneous groups of epithelial neoplasms originate in various anatomic locations, but are most prevalent in certain organs, such as lungs, stomach, appendix, cecum, duodenum, pancreas, jejunum/ileum, colon, and rectum, or they are diffused throughout the body, comprising nearly 0.49% of all malignancies (Fraenkel, Faggiano, & Valk, 2015; Singh & Law, 2012; Xiaojiang Yi, 2013). It is described that, two thirds of NETs arise and are detected in the gastroenteropancreatic system (Uccella, Sessa, & Rosa, 2015). Independently of their primary site and of their degree of differentiation, the incidence of NETs, currently estimated to be of 5.53/100,000 people/year for males and 4.76/100,000 people/year for females, has increased significantly in the last 30 years (Pusceddu et al., 2015).

NETs classification has been constantly evolving during the last decades. Nowadays, NETs comprise a broad spectrum of potentially malignant diseases spanning from rather benign

to very malignant and lethal variants, and many of them present hormonal syndromes and are highly vascularized solid tumors (Giandomenico, Thirlwell, & Essand, 2015; Uccella, Sessa, & Rosa, 2015; Yao et al., 2008). In the meanwhile, the understanding of NETs biology and treatment through different approaches based in exploring genetics, molecular pathways, molecular targets and potential novel biomarkers has changed dramatically in the last decade (Giandomenico, Thirlwell, & Essand, 2015). Latterly, one of the major limitations to overcome these malignancies relates to diversity in the mechanisms underlying the molecular pathogenesis of NETs. In cancer cells the signaling networks that tightly control the major cellular activities, such as proliferation, differentiation, apoptosis and survival are deregulated (Giandomenico, Thirlwell, & Essand, 2015). Regardless the current awareness, NETs diagnosis based on clinical symptoms, laboratory measurement of secretory hormones and peptides, radiologic and nuclear imaging, and histology, is often challenging (Singh & Law, 2012). Nevertheless, the knowledge of NETs biology has been going through a real revolution where new findings about specific molecular events have led to the development of novel molecular-targeted therapies against different targets that might become a feasible and better treatment alternative (Giandomenico, Thirlwell, & Essand, 2015). Despite all the progress in the knowledge about NETs biology there is a long way to unravel the key mechanisms for tumor progression.

1.2- Gastroenteropancreatic neuroendocrine tumors

Gastroenteropancreatic neuroendocrine tumors (GEP-NETs) comprise a group of rare and heterogeneous neoplasms that emerge from enterochromaffin epithelial cells of the diffuse endocrine system sparse throughout the mucosa of gastrointestinal tract and pancreas (Cigrovski Berkovic, Cacev, Catela Ivkovic, Zjacic-Rotkvic, & Kapitanovic, 2014). These cells share the structure and functional traits of the NE phenotype and have a wide spectrum of clinical features, which range from functioning tumors that might present with clinical/hormonal syndromes, to non-functioning tumors in terms of their capacity of synthetizing and secreting hormones and enzymes (Modlin et al., 2008; Modlin et al., 2010; Rindi & Wiedenmann, 2011). GEP-NETs also include a broad gamut of tumors, from very indolent to highly aggressive carcinomas (Uccella, Sessa, & Rosa, 2015). These solid tumors are characterized by a common neuroectodermal embryological origin and different clinical evolution displaying a broad spectrum of characteristics concerning behavior during growth and differentiation, functional aspects, localization and prognosis (Briest & Grabowski, 2014; Pusceddu et al., 2015). GEP-NETs represent 70% of all NETs and 2% of all digestive tract tumors (Modlin et al., 2008; Öberg, 2009; Singh & Law, 2012). Although

previously considered rare among neoplastic diseases in general, recent epidemiological studies have revealed that the incidence (3.6/100.000) and prevalence (35/100.000) of GEP-NETs has increased exponentially throughout the last decade, which has been attributed to the increased awareness, as well as, improved sensitivity of the imaging and endoscopic techniques employed. Currently, GEP-NETs are the second most common gastrointestinal malignancy after colorectal cancer (Cigrovski Berkovic, Cacev, Catela Ivkovic, Zjacic-Rotkvic, & Kapitanovic, 2014; Fraenkel, Kim, Faggiano, de Herder, & Valk, 2013; Meeker & Heaphy, 2014).

Since their first description in 1907 as carcinoid tumors or carcinoids, by the German physician and pathologist Siegfried Oberdorfer, these morphologically heterogeneous tumors have been a matter of debate in terms of their nomenclature and classification (Fraenkel, Faggiano, & Valk, 2015; Fraenkel, Kim, Faggiano, de Herder, & Valk, 2013; Meeker & Heaphy, 2014). In 1963, Williams and Sandler classified the GEP-NETs into three subcategories, based on embryological origin, as foregut (stomach, duodenum, upper jejunum and pancreas) tumors, midgut (lower jejunum, ileum, appendix and caecum) tumors and hindgut (colon and rectum) tumors (Meeker & Heaphy, 2014; Xiaojiang Yi, 2013). Later in 2000, the World Health Organization (WHO) defined a new classification introducing the terms neuroendocrine tumor (benign carcinoid) and neuroendocrine carcinoma (malignant carcinoid). However the term carcinoid, meaning cancer-like, despite being incorrect it is still traditionally used for some NE tumors (Klöppel, Perren, & Heitz, 2004; Meeker & Heaphy, 2014). Since 2010, the WHO/European Neuroendocrine Tumor Society (ENETS) new guidelines provided a useful and effective scheme to classify the GEP-NETs, based on the degree of histomorphological criteria and on proliferation rate (Blank, Schmitt, & Perren, 2015; Meeker & Heaphy, 2014; Xiaojiang Yi, 2013). According to this last classification, these tumors are classified in two different prognostic, diagnostic and biological broad categories: well-differentiated neuroendocrine tumors (WD-NETs) and poorly differentiated neuroendocrine carcinomas (PD-NEC) (Blank, Schmitt, & Perren, 2015; Pusceddu et al., 2015; Uccella, Sessa, & Rosa, 2015). A third category is represented by mixed endocrine-exocrine tumors, with both components, which are classified as mixed adenoneuroendocrine carcinomas (MANECs) (Blank, Schmitt, & Perren, 2015; Uccella, Sessa, & Rosa, 2015). This classification of GEP-NETs further separates WD-NETs into low-grade (G1) and intermediate grade (G2) categories, and PD-NECs into a high grade (G3) category (table 1). The tumor grade and classification is dependent on the proliferative behavior marked by the mitotic count and the Ki-67 proliferation index; G1, G2, and G3 tumors are defined as having a Ki-67 index of ≤2%, 3-20%, and >20%, respectively (table1) (Blank, Schmitt, & Perren, 2015; Briest & Grabowski, 2014; Pusceddu et al., 2015).

Table1- Classification and grading system of gastroenteropancreatic neuroendocrine tumors according to WHO/ENETS 2010 guidelines.

GEP-NETS nomenclature	Grading	Mitotic Count (10HPF)*	Ki-67 index (%)
WD-NETs	Grade 1 (G1)	<2	≤2
WD-NETs	Grade 2 (G2)	2-20	3-20
PD-NECs	Grade 3 (G3)	>20	>20

*10 HPF (high power fields): $2mm^2$ at least 40 fields

Despite the progress in understanding GEP-NETs, diagnosis is often challenging and complicated due to the high heterogeneity of the biological and clinical features: lack of symptoms in the early stages, high frequency of non-specific gastrointestinal symptoms and the absence of tumor markers (Cigrovski Berkovic, Cacev, Catela Ivkovic, Zjacic-Rotkvic, & Kapitanovic, 2014; Singh & Law, 2012; Uccella, Sessa, & Rosa, 2015). The delay in diagnosis often leads to patients presenting with advanced disease, often exhibiting metastases, and thus with a poor prognosis. Consequently, there is still a medical need for a better and earlier diagnosis, which is crucial for the starting point of the optimal treatment (Cigrovski Berkovic, Cacev, Catela Ivkovic, Zjacic-Rotkvic, & Kapitanovic, 2014; Singh & Law, 2012; Uccella, Sessa, & Rosa, 2015). For advanced disease, surgery, biotherapy, targeted therapies and chemotherapy are the best available thought limited therapeutic approaches to prolong survival but still not satisfactory, possibly due to the crosstalk in reactivate mitogen signaling. The only reliable curative treatment is early detection and surgical removal (Cigrovski Berkovic, Cacev, Catela Ivkovic, Zjacic-Rotkvic, & Kapitanovic, 2014; Giandomenico, Thirlwell, & Essand, 2015; Modlin et al., 2008; Rindi & Wiedenmann, 2011).

Chromogranin A (CgA), is expressed in 80-90% of patients with GEP-NETs together with synaptophysin, is the most useful and reliable serum tumor marker for the assessment of GEP-NETs however with some limitations (Blank, Schmitt, & Perren, 2015; Cigrovski Berkovic, Cacev, Catela Ivkovic, Zjacic-Rotkvic, & Kapitanovic, 2014; Singh & Law, 2012).The major problem that clinicians and scientists face in overcoming GEP-NETs is related to various phenomena within tumor biology and molecular pathogenesis. How signaling networks contribute to tumor progression and how these networks interact remains largely unclear, due to a lack of broad mechanistic knowledge and lack of research for novel predictive biomarkers. Therefore, there is an unmet need to identify unique biomarkers to improve the knowledge of GEP-NETs pathogenesis, to assess follow-up in terms of treatment efficacy, relapse and prognosis, as well for developing and identifying

the best therapeutic options (Briest & Grabowski, 2014; Cigrovski Berkovic, Cacev, Catela Ivkovic, Zjacic-Rotkvic, & Kapitanovic, 2014; Giandomenico, Thirlwell, & Essand, 2015).

1.3- Metabolic Syndrome

Metabolic syndrome (MS) is a cluster of interconnected clinical and biochemical factors that predict the increased risk of atherosclerotic cardiovascular disease, type 2 diabetes (T2D) and mortality. These risk factors incorporate central obesity, high blood pressure (BP), raised triglycerides, low high-density lipoprotein (HDL) and high fasting plasma glucose (Alberti et al., 2009; Esposito, Chiodini, Colao, Lenzi, & Giugliano, 2012; Giugliano, Ceriello, & Esposito, 2006; Kaur, 2014). MS initial concept was described in 1920 by a Swedish physician named Kylin when the association between high BP, high fasting plasma glucose and gout was described. Later in 1988, Reaven was responsible for the revival and progress of MS when he postulated a "cluster of risk factors for diabetes and cardiovascular diseases" that was designated by "Syndrome X" (Hanefeld, Pistrosch, Bornstein, & Birkenfeld, 2016; Kaur, 2014). Since then, a wide variety of MS clinical definitions have appeared throughout times with common and diverse risk factors which led to some confusion and discussion. Nowadays, MS is a common medical term with a concrete diagnosis, but is not well recognized as a clinical entity. The most recent criteria used to diagnose MS are from a jointly effort of several institutions (Alberti et al., 2009; Kaur, 2014). According to this joint scientific statement, MS is defined by the presence of at least three out of the five risk factors: diabetes or high fasting plasma glucose, central obesity/visceral obesity, raised triglycerides, low HDL and high BP (Alberti et al., 2009). The criteria and respective reference values are showed in table 2. It is estimated that people with MS are twice as likely to die, three times as likely to have a myocardial infarction or stroke and a five-fold greater risk of developing T2D, when compared with people without the syndrome (Alberti et al., 2009; Kaur, 2014).

Table 2- Criteria for MS clinical diagnose and definition (adapted from Alberti et al., 2009).

Criteria	Reference Values	Additional information
Central obesity (defined as waist circumference*) *with ethnicity specific values	**≥ 94 cm (increased risk) and ≥ 102 cm (still higher risk) in European males Or ≥ 80 cm (increased risk) and ≥ 88 cm (still higher risk) in European females**	if BMI is >30kg/m², central obesity can be assumed.
Raised triglycerides	**≥ 150 mg/dL**	or specific treatment for this anomaly
Low HDL	**< 40 mg/dL in males or < 50 mg/dL in females**	or specific treatment for this anomaly
High blood pressure	**Systolic BP ≥ 130 mm Hg or Diastolic BP ≥ 85 mm Hg**	or specific treatment for this anomaly
High fasting plasma glucose	**≥ 100 mg/dL**	or previously diagnose T2D

In parallel with the increasing rate of GEP-NETs, obesity, MS and diabetes are also major and escalating public-health and clinical problems worldwide (Kaur, 2014; Vigneri, Frasca, Sciacca, Pandini, & Vigneri, 2009; Zeyda & Stulnig, 2009). The International Diabetes Federation estimates that a quarter of the world's adult population has the MS and that it is starting to be a serious ubiquitous health concern throughout the world. It is reported that worldwide prevalence of MS ranges from <10% to as much as 84%, depending on the region, urban or rural environment, composition (sex, age, race, and ethnicity) of the population studied (Esposito, Chiodini, Colao, Lenzi, & Giugliano, 2012; Kaur, 2014). In United States, MS is highly prevalent and affects more than 35% of the population, which is translated in nearly 80 million adults affected (Esposito, Chiodini, Colao, Lenzi, & Giugliano, 2012; Giugliano, Ceriello, & Esposito, 2006). MS is also consistently associated with an increased risk and mortality of several cancers in adults and for some cancers the risk differs between sexes and populations (Esposito, Chiodini, Colao, Lenzi, & Giugliano, 2012). Some studies also revealed the correlation between MS individual components and cancer risk. Obesity and diabetes, contributing factors for MS prevalence and risk, have frequently been correlated with a raised risk for several types of cancers, such as gastric

adenocarcinoma, pancreatic, colorectal, liver, breast and esophageal cancer among others (Esposito, Chiodini, Colao, Lenzi, & Giugliano, 2012; Lin, Ness-Jensen, Hveem, Lagergren, & Lu, 2015; Vigneri, Frasca, Sciacca, Pandini, & Vigneri, 2009). Despite the available facts, the precise etiology and molecular mechanisms that connect MS to the development, progression and cancer mortality are not entirely understood (Esposito, Chiodini, Colao, Lenzi, & Giugliano, 2012). The link between MS, its components and cancer has been recently demonstrated for several types of neoplasia, however the association with NETs has not so far been reported, although it deserves to be explored.

Obesity, diabetes, and MS are closely related with a low grade inflammatory state (Giugliano, Ceriello, & Esposito, 2006; Vigneri, Frasca, Sciacca, Pandini, & Vigneri, 2009; Zeyda & Stulnig, 2009). MS is a state of chronic low grade inflammation due to the contribution of the interaction among components of the clinical syndrome phenotype with its biological phenotype, resulting in deep systemic effects (Hanefeld, Pistrosch, Bornstein, & Birkenfeld, 2016; Kaur, 2014). Since a long time an association between chronic inflammation, visceral obesity and insulin resistance has been known. This association is characterized by abnormal production of inflammatory cytokines, such as, tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8), leptin, osteopontin and adiponectin by both adipocytes and infiltrate immune cells, resulting in a pro-tumorigenic environment and elevated plasma levels (Esposito, Chiodini, Colao, Lenzi, & Giugliano, 2012; Kaur, 2014; Zeyda & Stulnig, 2009). Recent studies indicate that chronic low-grade inflammation plays a key role in metabolic deterioration in the obese population, where adipose tissue is as the key source for increased pro-inflammatory cytokines during obesity and circulating monocyte/macrophages in adipose tissue contribute to both the pro-inflammatory state and insulin resistance of MS. So these studies suggest that obesity associated systemic chronic low grade inflammation is a major risk factor for the development of MS and associated health complications (Esposito, Chiodini, Colao, Lenzi, & Giugliano, 2012; Sun, Ji, Kersten, & Qi, 2012; Zeyda & Stulnig, 2009). As a result of a complex interplay between genetic and environmental factors MS is a state of chronic low grade inflammation. However, the mechanisms of origin pathophysiology and mode of perpetuation of such inflammation are not quite completely known (Kaur, 2014).

1.4- Inflammation and cancer

A causal relationship between inflammation and cancer has been suspected for a long time, due to the contribution of Galen (Greten & Karin, 2004). Since the early pathology studies in the 1800s, Rudolf Virchow demonstrated the presence of leucocytes in malignant tissues

claiming that tumors arise from regions of chronic inflammation, thus providing the first evidence between inflammation and cancer (Balkwill & Mantovani, 2001; Greten & Karin, 2004; Grivennikov, Greten, & Karin, 2010). However, it was only during last decades that became evident that inflammation affects immune surveillance, response to therapy and plays a vital role in tumorigenesis. Therefore, the importance of the inflammatory response in tumor initiation and malignant progression has assumed prominence enabling that the mechanisms that link infection, innate immunity, inflammation, and cancer be revealed at a fast pace (Cigrovski Berkovic, Cacev, Catela Ivkovic, Zjacic-Rotkvic, & Kapitanovic, 2014; Grivennikov, Greten, & Karin, 2010; Lin & Karin, 2007; Yu, Pardoll, & Jove, 2009). Inflammatory response is in many aspects similar with a regenerative or scaring response, although subverted, and in the past tumors have been considered as wound that do not heal (Balkwill & Mantovani, 2001; Grivennikov, Greten, & Karin, 2010). Despite this increasing knowledge, the exact molecular nature of the link between inflammation and cancer remains somewhat vague and needs to be further characterized (Greten & Karin, 2004).

It has become clear that diverse types of inflammation can promote or inhibit the induction and growth of cancer. As seen in inflammatory bowel disease (IBD) and chronic pancreatitis an elevated risk is respectively associated with colorectal cancer or pancreatic adenocarcinoma, demonstrating that the carcinogenesis in the gastrointestinal tract and pancreas is often associated with chronic inflammation (Cigrovski Berkovic, Cacev, Catela Ivkovic, Zjacic-Rotkvic, & Kapitanovic, 2014; Grivennikov, Greten, & Karin, 2010). As a matter of fact, several human cancers arise and progress under conditions of a chronic inflammatory state, once chronic inflammation orchestrates a microenvironment that is an indispensable part in the neoplastic process (Grivennikov, Greten, & Karin, 2010; Lin & Karin, 2007). Although chronic inflammation increases cancer risk, the exact molecular mechanism by which promotes tumor is not yet entirely known and therefore understood (Cigrovski Berkovic, Cacev, Catela Ivkovic, Zjacic-Rotkvic, & Kapitanovic, 2014).

The aforementioned inflammatory response can stimulate tumorigenesis at different stages, initiation, promotion, malignant transformation, invasion and metastasizing, while inflammation, mainly acute inflammation, together with immunity is able to inhibit tumor growth and even also to eliminate some tumors (Cigrovski Berkovic, Cacev, Catela Ivkovic, Zjacic-Rotkvic, & Kapitanovic, 2014; Grivennikov, Greten, & Karin, 2010; Lin & Karin, 2007). Nowadays has become evident that an inflammatory microenvironment is an essential component of all tumors, even though in some tumors a direct causal link is not yet proved. In the tumor microenvironment, there is a delicate balance between anti-tumor immunity and pro-inflammatory activity (Grivennikov, Greten, & Karin, 2010; Lin & Karin, 2007). The

direction in which the balance is tipped depends of growth factors, matrix degrading enzymes, reactive oxygen species (ROS) and the expression of different existing mediators, like cytokines, chemokines among others, in tumor microenvironment, which are released by host immune and inflammatory cells, cancer cells, and other types of host tumor associated cells, like fibroblasts and endothelial cells (Cigrovski Berkovic, Cacev, Catela Ivkovic, Zjacic-Rotkvic, & Kapitanovic, 2014; Coussens & Werb, 2002; Lin & Karin, 2007). Immune cells that infiltrate the tumor and other surrounding cells are involved in an extensive and dynamic crosstalk with tumor cells (Grivennikov, Greten, & Karin, 2010). Genetic and epigenetic alterations in malignant cells also can generate an inflammatory microenvironment supporting tumor progression. So, during tumorigenesis the host-mediated anti-tumor immunity is suppressed and at the same time pro-inflammatory activity is favored and thus leading to tumor development (Cigrovski Berkovic, Cacev, Catela Ivkovic, Zjacic-Rotkvic, & Kapitanovic, 2014).

Regarding the different mediators present in tumor microenvironment it is most important to highlight that cytokines are the main mediators responsible for shaping the microenvironment. The cytokines profile predominantly expressed in tumor microenvironment is able either to favor pro-inflammatory activity, promoting tumor growth, or anti-tumor activity, suppressing tumor growth, by controlling the direction in which the delicate balance is tilted (Cigrovski Berkovic, Cacev, Catela Ivkovic, Zjacic-Rotkvic, & Kapitanovic, 2014; Lin & Karin, 2007). Usually, independently of their source cytokines are divided in two main groups: pro-inflammatory group, IL-1, IL-6, IL-8, interleukin-11, interleukin-12 (IL-12), interleukin-18, interleukin-23 (IL-23), TNF-α, among others, which can contribute to tumor development and progression and the anti-inflammatory group, interleukin-4, interleukin-10 (IL-10), interferon-alpha, interferon-beta, transforming growth factor-beta (TGF-β), among others, which can contribute to the inhibition of tumor development and progression (Cigrovski Berkovic, Cacev, Catela Ivkovic, Zjacic-Rotkvic, & Kapitanovic, 2014; Grivennikov, Greten, & Karin, 2010). In both of these groups, certain cytokines, like TNF-α, interleukin-1 alpha, interleukin-1 beta (IL-1β), IL-6, IL-10, IL-12, interleukin-17 (IL-17), TGF-β and IL-23, are associated with fostering a particular direction, although some cytokines can have a bifold performance (Cigrovski Berkovic, Cacev, Catela Ivkovic, Zjacic-Rotkvic, & Kapitanovic, 2014; Lin & Karin, 2007). Indeed, some studies have been consistently revealing and associating a crucial role of TNF-α, IL-6, IL-1β and IL-17 in tipping the balance into pro-inflammatory activity and thus in the initiation of chronic inflammation, resulting in a support to tumor growth and progression via the stimulation of different pathways and several downstream effectors (Coussens & Werb, 2002; Grivennikov, Greten, & Karin, 2010; Lin & Karin, 2007). The activation with different

mediators of nuclear factor-kappa B (NF-kB) and signal transducers and activator of transcription 3 (STAT3) pathways are crucial for the initiation, progression, tumor-promoting inflammation and survival of several cancers. The persistence activation of NF-kB and STAT3 signaling pathways in multiple cancers is due to a process of an autocrine or paracrine loop. These feed-forward loops are established through a tumor associated inflammation that upregulate mediators capable to attract immune and inflammatory cells that further propagate the pathways activity by the production of different mediators, mainly IL-6 and IL-1β, or due to the control and induction of the transcription of genes that encode pro-inflammatory cytokines, such as TNF-α and IL-6 (Cigrovski Berkovic, Cacev, Catela Ivkovic, Zjacic-Rotkvic, & Kapitanovic, 2014; Greten et al., 2004;Yu, Pardoll, & Jove, 2009).

Recent reports have revealed that there is a considerable higher incidence of a chronic inflammatory environment in GEP-NETs. For instance, research have shown an increased risk for developing of gastrointestinal neuroendocrine tumors in IBD. It has been hypothesized that chronic inflammatory conditions stimulate enteroendocrine cells and consequently leading to hyperplasia and possibly to neoplastic transformation (Cigrovski Berkovic, Cacev, Catela Ivkovic, Zjacic-Rotkvic, & Kapitanovic, 2014; Le Marc'Hadour et al., 1994). Studies pinpoint a role for cytokines in NETs, suggesting that IL-1 and interleukin-2 could have role in alterations, differentiation and regulation of a neuroendocrine system and in the interaction with immune system (Qian, El-Salhy, Melgar, Hammarstrom, & Danielsson, 2000; Sun, 2004). GEP-NETs express a variety of mediators including a diverse group of growth factors, cytokines, enzymes and tyrosine kinase receptors that are used as targets for the development of new therapies (Cigrovski Berkovic, Cacev, Catela Ivkovic, Zjacic-Rotkvic, & Kapitanovic, 2014). Nowadays, the influential role of inflammation in tumorigenesis is widely accepted, and it has become evident that an inflammatory microenvironment is an essential component of all tumors (Grivennikov, Greten, & Karin, 2010). Despite that, this role and association of inflammation and tumor microenvironment in GEP-NETs is still not completely understood.

1.5- Cellular signaling and NETs

The understanding of GEP-NETs signaling pathways, in terms of their molecular mechanisms and interactions, is still far from a sharp molecular awareness (Briest & Grabowski, 2014). It is known that cell signaling in NETs influence nearly all aspect of cancer pathophysiology, such as malignant transformation, progression and metastasis, through the activation of several signaling pathways. Generally, NETs signaling activation involves the activation of several receptors, such as tyrosine kinase receptors (TKRs) and

G-protein coupled receptors, leading to the activation of downstream cell signaling cascades and resulting in an increased DNA synthesis, angiogenesis, cell proliferation, survival, migration, growth and secretion (Figure 1). Receptor activation is due to the linking of their ligands, mainly growth factors. GEP-NETs express a wide variety of growth factors, vascular endothelial growth factor (VEGF), platelet-derived growth factor, insulin-like growth factor 1 (IGF-1), transforming growth factor, somatostatin among many others and *in vitro* studies, suggest that tumor growth depends on the activation of some growth factor receptors. Of the several signaling networks those considered to be the most important in neuroendocrine biology are phosphatidylinositol 3-kinase (PI3k)/AKT/mammalian target of rapamycin (mTOR), RAS/RAF/ mitogen-activated protein kinase (MAPK), phospholipase C-protein kinase C (PLC/PKC), and Janus kinase–signal transducer and activator of transcription (JAK/STAT) (Figure 1) (Briest & Grabowski, 2014; Giandomenico, Thirlwell, & Essand, 2015; Raymond et al., 2011).

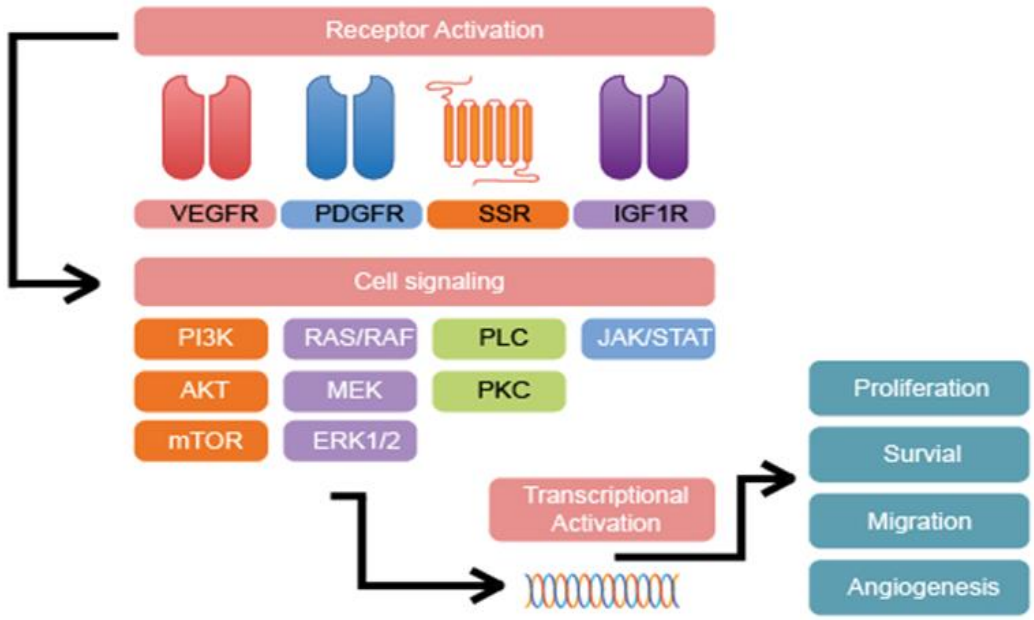


Figure 1- Neuroendocrine cell signalization and involved pathways (adapted from Raymond et al., 2011).

The major pathways involved in neuroendocrine tumor biology are MAPK and PI3K-Akt-mTOR. The MAPK signaling pathway has been identified and amply studied for more than three decades, but researchers are still puzzled by the intricate dynamic control and plasticity of its kinases (Fey, Matallanas, Rauch, Rukhlenko, & Kholodenko, 2016; Rauch, Rukhlenko, Kolch, & Kholodenko, 2016). This cascade is a pivotal signaling pathway that

comprises several ubiquitously expressed kinases, from membrane to nucleus, that regulate a wide variety of cellular functions, including cell proliferation, growth, differentiation, survival/apoptosis, migration, transformation, metabolism, transcription and translation, in response to a plethora of internal/external signals, such as growth factors, cytokines, chemokines, extracellular matrix, ROS, drugs among many others (Briest & Grabowski, 2014; Cuevas, Abell, & Johnson, 2007; Rauch, Rukhlenko, Kolch, & Kholodenko, 2016). Enduring temporal alterations of the MAPK cascade has been frequently associated with a key role in multiple diseases. In truth, MAPK cascade is one of the most frequently affected pathways and their deregulation is associated with several cancers, development disorders and other human diseases, including inflammatory and proliferative diseases (Cuevas, Abell, & Johnson, 2007; Fey, Matallanas, Rauch, Rukhlenko, & Kholodenko, 2016; Rauch, Rukhlenko, Kolch, & Kholodenko, 2016).

The MAPK pathway is constituted by different cascades, being the classic cascade one of the most important pathways (ERK/MAPK pathway). This classic cascade is induced by ligand binding to TKRs, such as vascular endothelial growth factor receptor, epidermal growth factor receptor or insulin-like growth factor receptor, leading to the sequential phosphorylation of specific substrate proteins of a dynamic protein kinase network which are frequently mutated in cancer and are drug targets (Briest & Grabowski, 2014; Rauch, Rukhlenko, Kolch, & Kholodenko, 2016). MAPK pathways are associated with STAT3 phosphorylation leading to the stimulation of cytokine production, induction of pro-angiogenic factors and invasion in tumor cells (Briest & Grabowski, 2014). Multiple crosstalks and feedback loops, both positive and negative, between pathways brings a complex dynamic inducing resistance to therapy and bypass inhibitory approaches (Fey, Matallanas, Rauch, Rukhlenko, & Kholodenko, 2016; Rauch, Rukhlenko, Kolch, & Kholodenko, 2016). In NETs, the MAPK pathway is highly activated, although the activation mechanisms is not clear, contributes to neuroendocrine tumorigenesis and is highly involved in the triggering of a neuroendocrine phenotype (Briest & Grabowski, 2014).

The unique ubiquitous PI3K signaling pathway is frequently activated in several human cancers by different mechanisms and is one of the fundamental pathways involved in cancer development and preservation (Wong, Engelman, & Cantley, 2010; Yuan & Cantley, 2008). PI3K activation is carefully regulated by the interactions between ligands, such as growth factors, and TKRs that directly or indirectly activate the downstream signaling cascade (Wong, Engelman, & Cantley, 2010; Yuan & Cantley, 2008). TKRs integrate and propagate the signal from the extracellular growth factors, like VEGF, IGF-1, fibroblast growth factor, to effector proteins of the intracellular signaling cascade that play a critical role in controlling a wide range of processes, such as cellular growth, proliferation, motility,

survival, insulin signaling, signaling with endothelial and immune cells, inflammation, redox status, regulation of membrane trafficking among many other functions (Briest & Grabowski, 2014; Yuan & Cantley, 2008). The PI3K signaling pathway is generally highly activated and deregulated in GEP-NETs, due to vastly expression levels of diverse growth factors and the contribution of several feedback loops (Briest & Grabowski, 2014).

The most promising therapies in GEP-NETs are molecular targeted approaches, targeting mTOR protein and somatostatin receptors (Briest & Grabowski, 2014). In insulin resistance states, the high insulin levels are known to stimulate MAPK and PI3K-Akt-mTOR pathways, which are attenuated by metformin. Metformin lowers circulating insulin levels and indirectly stimulates AMP-activated protein kinase activity leading to a reduction of insulin/IGF1 signaling, and thus suppressing MAPK and mTOR signaling. In addition, metformin has also demonstrated anti-cancer properties, through the inhibition of cell growth and signaling, on neuroendocrine *cells in vitro* (Vigneri, Frasca, Sciacca, Pandini, & Vigneri, 2009; Vlotides et al., 2014).

Recent evidence allowed to disclosure the complicated and multifactorial role of the insulin-like growth factor one receptor (IGF1R) pathway in the development and progression of several tumors. More specifically, increased expression of IGF1 and IGF1R has been involved in the progression of different types of tumors, including breast, lung and endocrine tumors among others (Raymond et al., 2011; Samani, Yakar, LeRoith, & Brodt, 2007). IGF1R is one of the crucial TKRs in GEP-NETs biology, where the activation and participation of all components of the IGF are necessary for the occurrence of the tumorigenic process (Briest & Grabowski, 2014; Raymond et al., 2011). In physiological conditions, IGF1R expression is under a tightly balanced control and any disturbance can induce the activation of a downstream molecular cascade. The increase expression of both IGF1R and IGF1 by different tumors, including gastrinomas, were associated with tumor growth, aggressiveness, decreased survival and poor prognosis (Furukawa et al., 2005; Raymond et al., 2011; Samani, Yakar, LeRoith, & Brodt, 2007). Neuroendocrine cells that secrete a large amount of IGF-1, lead to IGF1R activation and consequently to a high expression of these TKRs and its ligand (Briest & Grabowski, 2014). IGF-1 and insulin are weak activators of MAPK pathway but strong activators of the PI3K-Akt-mTOR pathway (Raymond et al., 2011). It was also demonstrated that IGF1 can be a major autocrine regulator of neuroendocrine tumor growth and secretion through the activation of complex molecular networks (Briest & Grabowski, 2014). IGF receptors are potential molecular targets for a variety of therapies in GEP-NETs and several inhibitors and monoclonal antibodies for IGFR1 are currently under trial (Briest & Grabowski, 2014; Raymond et al., 2011).

The *Forkhead box M1* (FOXM1) is a common proliferation-associated transcription factor, which is a member of the *forkhead box* (FOX) transcription superfamily that regulates several biologic processes (Gomes, Zhao, & Lam, 2013; Halasi & Gartel, 2013). FOXM1 is an essential transcription factor in neoplastic cells of a variety of human solid cancers, including GEP-NETs, and is usually associated with cell cycle progression, cell development, differentiation, proliferation, apoptosis, tissue homeostasis and angiogenesis (Briest et al., 2015; Gomes, Zhao, & Lam, 2013; Halasi & Gartel, 2013). This transcription factor is one of the first's up-regulated proteins in tumorigenesis, with an important function in tissue repair, DNA replication and mitosis, and their role in all of the hallmarks of cancer has been proposed and demonstrated (Gomes, Zhao, & Lam, 2013; Halasi & Gartel, 2013; Wierstra, 2013). More specifically, FOXM1 regulates the expression of cell cycle genes, whose products control the transitional progress of the G1/S-phase G2/M-phase cell cycle checkpoints and are associated with the regulation of mitotic spindle integrity for a proper mitosis (Halasi & Gartel, 2013; Halasi & Gartel, 2013; Wierstra, 2013). FOXM1 transcription factor belongs to the PI3K-Akt-Forkhead Box O (FOXO) pathway, where it is an important downstream effector of this cascade that can be repressed by wild type p53 or Forkhead O3a (FOXO3a) (Gomes, Zhao, & Lam, 2013; Halasi & Gartel, 2013; Wierstra, 2013). FOXM1 is a transcriptional target of STAT3 and in GEP-NETs they are unitedly upregulated. This pathway is also commonly deregulated in gastrointestinal NETs (Briest et al., 2015; Wierstra, 2013). MAPK and PI3K pathways, the most frequently deregulated pathways in cancer, establish a crosstalk with FOXM1 pathway (Halasi & Gartel, 2013; Wierstra, 2013). In a study, FOXM1 expression was strongly correlated with tumor differentiation, proliferation and metastasis suggesting that could possibly be used as a prognostic factor and as a therapeutic target for gastrointestinal NETs (Briest et al., 2015). A study demonstrated a correlation between FOXM1 and Ki-67 and that FOXM1 were predominantly expressed in high grade neuroendocrine tumors, G2 and G3 tumors, than in carcinoid tumors (Briest et al., 2015). The expression of some proteins, like IGF-1, is altered in GEP-NETs and a study in cardiomyocytes showed that a downregulated FOXM1 expression is associated with a downregulated IGF1R expression (Briest & Grabowski, 2014; Sengupta, Kalinichenko, & Yutzey, 2012). The overexpression of this crucial factor promotes resistance to chemotherapy and other genotoxic drugs of several human cancers leading to cancer cell growth and survival (Gomes, Zhao, & Lam, 2013; Halasi & Gartel, 2013; Halasi & Gartel, 2013). Furthermore, studies with siomycin A, an inhibitor of FOXM1, in GEP-NETs and metastatic melanoma cell lines *in vitro* induced a decreased in the mitotic activity, due to a down-regulation of mitotic proteins that results in mitotic catastrophe, inducing apoptosis and presenting synergic effects with conventional chemotherapy (Bhat, Zipfel, Tyler, & Gartel, 2008; Briest et al., 2015). Although the increasing knowledge, there

is still a need for a better understanding of FOXM1 role and mechanistic action in NETs and further studies need to be done.

Chapter 2- Objective

The main objective of this project was to evaluate the influence of metabolic syndrome criteria and their individual parameters in the expression of different molecular markers that participate in metabolic and inflammatory pathways in neuroendocrine tumors and peri-tumoral tissue, as well as, their correlation with the tumor clinical and pathologic features.

Chapter 3- Materials and Methods

3.1- Human tumor samples

Paraffin-embedded neuroendocrine tumor tissue (n= 39) collected from patients under routine follow up at the Endocrine Tumors Clinic of the Portuguese Institute of Oncology in Porto were selected and used. These selection only included patients with NETs from the gastroenteropancreatic system with the demographics and tumor pathological features presented in table 3 and table 4. In table 3, the divergence between number of patients and sum of studied parameters translates missing data.

Table 3- Characterization of patients and tumors characteristics.

Parameters	Pancreas (n=10)	Gastrointestinal (n=29)
Median Age, **years (range)**	57 (29-75)	64 (41-81)
Sex **F:M**	6:4	12:17
WHO grade **G1:G2**	6:4	24:5
T T1:T2:T3:T4	3:3:1:2	4:6:9:7
N N0:N1	8:1	5:19
M M0:M1	7:3	9:20
Functioning Yes:No	2:7	22:7
GI-NETs location		Duodenum (n=4), Ampoule (n=1) Jejunum (n=1), Ileum (n=20), Appendix (n=3)

Table 4- Characterization of patients characteristics in terms of MS and its parameters.

Parameters	Pancreas (n=10)	Gastrointestinal (n=29)
MS Present:Absent	6:4	20:9
Tumor grade MS Present:Absent	G1- 4:2 G2- 2:2	G1- 17:7 G2- 3:2
Waist circumference Normal:Raised	3:6	12:17
BP Normal:Raised	4:6	9:20
Triglycerides Normal:Raised	4:6	16:13
HDL Normal:Decreased	5:5	13:16
High fasting plasma glucose Normal:Raised	4:6	9:20

3.2- Immunohistochemistry

In order to evaluate the inflammatory state of the tumors adjacent tissue and understand the influence of MAPK and mTOR pathways in the biology of neuroendocrine tumors immunohistochemical analysis were performed. Therefore individual analysis for the presence of IL-6, FOXM1 and IGF1R proteins were respectively accomplished.

For this immunohistochemistry study the positive and negative internal controls used were breast cancer (IGF1R), colon (FOXM1) and tonsil tissue (IL-6).

The tissue sections of 3 µm were dewaxed in xylene and progressively rehydrated in a decreasing scale of alcohols (100%, 95% and 70%) up until to water. Antigenic retrieval was done differently according to the different types of markers molecules. For IL-6 antigen retrieval was made by incubation in a 10 mM citrate buffer (pH 6,0) with Tween 20 at 0,05%, in a microwave at 900 W during 20 minutes after boiling. For FOXM1 antigen retrieval was made by incubation in a 10 mM citrate buffer (pH 6,0) with Tween 20 at 0,05%, in a microwave at 900 W during 25 minutes after boiling. For IGF1R protein, antigen retrieval was made by incubation in a 10 mM citrate buffer (pH 6,0) with Tween 20 at 0,05%, in a pressure cooker during 4 minutes after boiling. All of the washes required throughout the process were performed with a phosphate buffered saline solution with Tween 20 at 0,05% (pH 7,4). Endogenous peroxidase was inhibited with the incubation of the sections in a

solution of hydrogen peroxide and methanol at 3% during 20 minutes. Slides were then mounted in the Sequenza Immunostaining Center (Thermo Scientific Shandon) and the sections were incubated with a serum solution (dilution 1:5 in BSA 10%) to ensure the blocking of unspecific marking, during 30 minutes. For the different proteins different serums were used: rabbit serum for IL-6 and swine serum for FOXM1 and IGF1-R.

Incubation with the respective primary antibody, was performed overnight at 4ºC in the conditions described in the table 5. The negative control was incubated only with 5% BSA.

Table 5- Characteristics and dilutions of primary antibodies.

Primary Antibody	Anti-IGF1R	Anti-FOXM1	Anti-IL-6
Brand and reference	Abcam Ref: ab39675	Santa Cruz Biotechnology Ref: (C-20): sc-502	Abcam Ref: ab9324
Dilution in 5% BSA	1:100	1:500	1:500

Subsequently the sections were incubated with the proper secondary antibody (Table 6) during 30 minutes at room temperature.

Table 6- Characteristics and dilutions of secondary antibodies.

Secondary Antibody	Polyclonal Rabbit anti-Mouse Biotinylated	Polyclonal Swine anti-Rabbit Biotinylated
Brand and Reference	Dako Cytomation E0354	Dako Cytomation E0353
Dilution in 5% BSA	1:200	1:200
Used against	IL-6	FOXM1 and IGF1R

After that, sections were incubated during 30 minutes with an avidin-biotin complex (Vector A and B, in a 1:100 dilution in BSA 5%) and then revealed with the DAB substrate (3,3'-Diaminobenzidine tetrahydrochloride, Dako) during different times for the different antibodies (IL-6 (30 seconds), FOXM (1minute), IGF1R (2 minutes)). The revelation process was stopped with running water, 10 minutes, after which the sections were counterstained with Harris Hematoxylin, three dives, followed by a 10 minute wash in running water. Finally, the blades with the sections were progressively dehydrated in an increasing scale of alcohols (70%, 95% and 100%), cleared with xylene and mounted with Entellan.

Tissue slides immunohistochemically stained for the Ki-67 marker routinely performed to determine the tumor grade were retrieved from the IPO-PORTO pathology department archives and used for morphological analysis.

3.3- Immunohistochemical data analysis

Hematoxylin-Eosin stained slides were used for tumor area delimitation based in morphologic criteria by the same experienced pathologist with no access to patients' clinical information. This area delimitation was then transferred to the immunohistochemistry stained slides.

After the immunohistochemistry, slides were scanned using the image acquisition Olympus VS110 virtual slide scanning system and captured with a magnification of 20x using the image acquisition software VS-ASW. Images were analyzed using the image processing software FIJI (Life Line Version from June 2nd 2014 for Macintosh, National Institutes of Health – USA).

The neuroendocrine tumor area was selected using Fiji freehand tool, for the study of the expression of Ki-67, FOXM1 and IGF1R. A peri-tumoral area 5mm distant from the tumor and from 5mm till the end of the tissue was delimited using a macro for Fiji developed by Professor Paula Sampaio (I3S, IBMC) to evaluate IL-6 expression. This macro is based in the ROI Manager Tool of FIJI.

Using FIJI color deconvolution plugin (H Dab), which allows the separation of the stained area from the initial image, based in the RGB system (Red, Green and Blue), the stained area with the antibodies in the total tissue area of the tumor and adjacent tissue were quantified.

Using just one of the images provided by the color deconvolution (red) the threshold command was used to transform the image in a black and white binary system allowing to quantify the stained area (brown staining with Dab) for the different analyzed regions. Then the Fiji command analyze particles was selected to introduce the results (total stained area). Each one of the individual starting images was duplicated and converted in a 16 bits format (shades of gray). Using this converted 16 bits image a new threshold command was applied in order to allow to quantify the total area for the different analyzed regions. After that the option analyze particles was chosen to show the results of interest (total area). Finally, the percentage of the stained area for the different analyzed regions (Neuroendocrine tumor- Ki-67, IGF1R and FOXM1; tumors adjacent tissue- IL-6) was assessed by calculating the ratio between the antibody stained area in these regions and the respective total area.

3.4- Correlation of the immunohistochemical results with the patients and tumor characteristics

After the immunohistochemical analysis, the results were correlated with metabolic syndrome and its components. More specifically the criteria selected for the correlation with immunohistochemistry were presence or absence of MS, normal and abnormal levels of waist circumference, HDL, triglycerides, BP, high fasting plasma glucose (MS components).

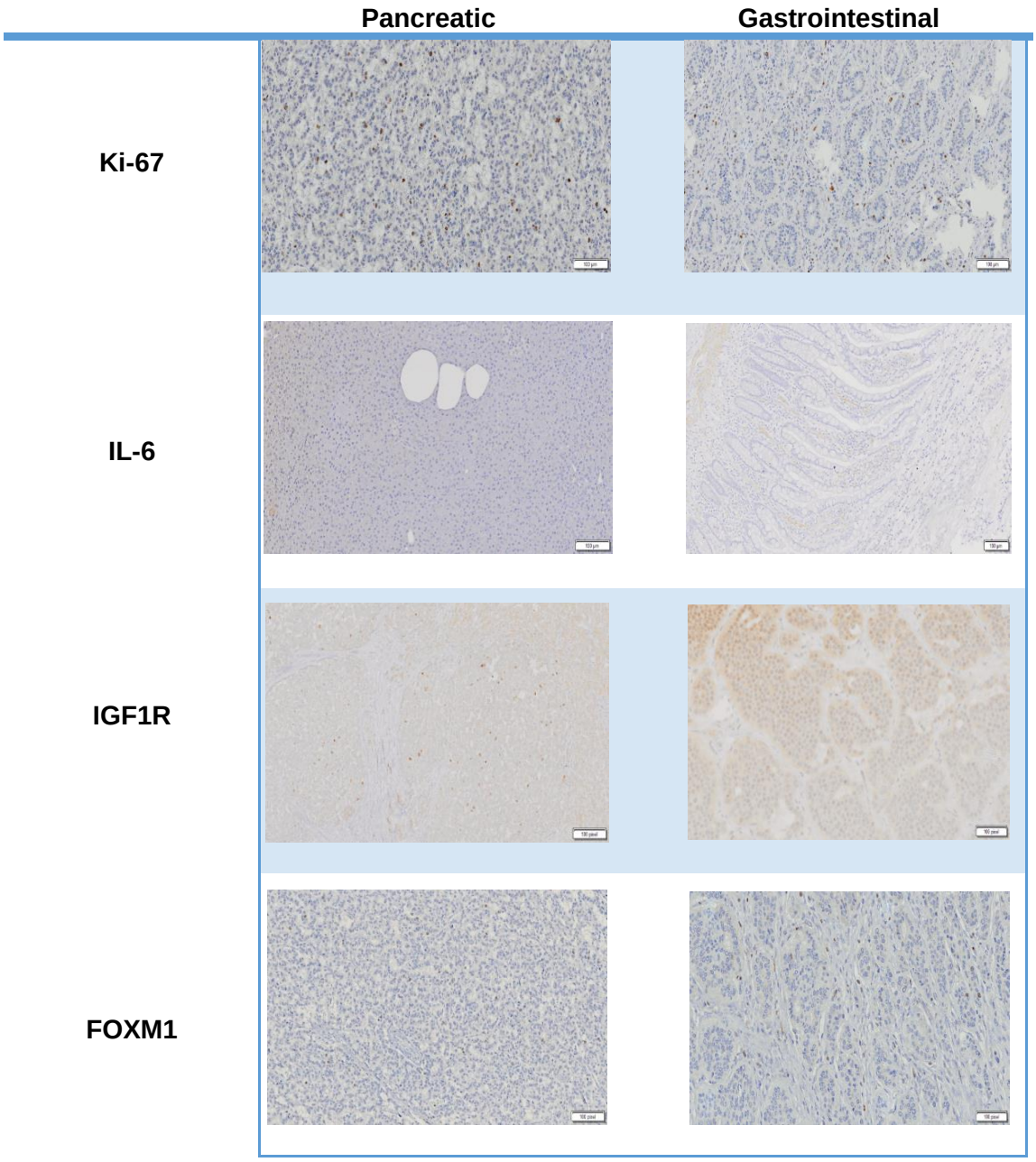
3.5- Statistical analysis

Qualitative variables are expressed as number of cases and percentage (%), and the quantitative variables are expressed as mean and standard error of the mean. The difference between two independent experimental groups was evaluated using the unpaired Student t test for normally distributed variables, and the Mann-Whitney U test for variables that did not meet the normal parameters. To compare 3 or more independent groups with normal distribution we used a simple analysis of variance (one-way ANOVA) with post-hoc Newman Keuls test. Kruskal-Wallis ANOVA with Dunns post hoc was used to compare 3 or more groups when a sample did not meet the criteria of normality. To correlate the different groups, a Pearson or Spearman correlation was used as the normality of the samples. A p value <0.05 was considered statistically significant. All statistical analyses were performed with the aid of the Graphpad Prism software version 7.00 and IBM SPSS Statistics version 24, both for Windows.

Chapter 4- Results

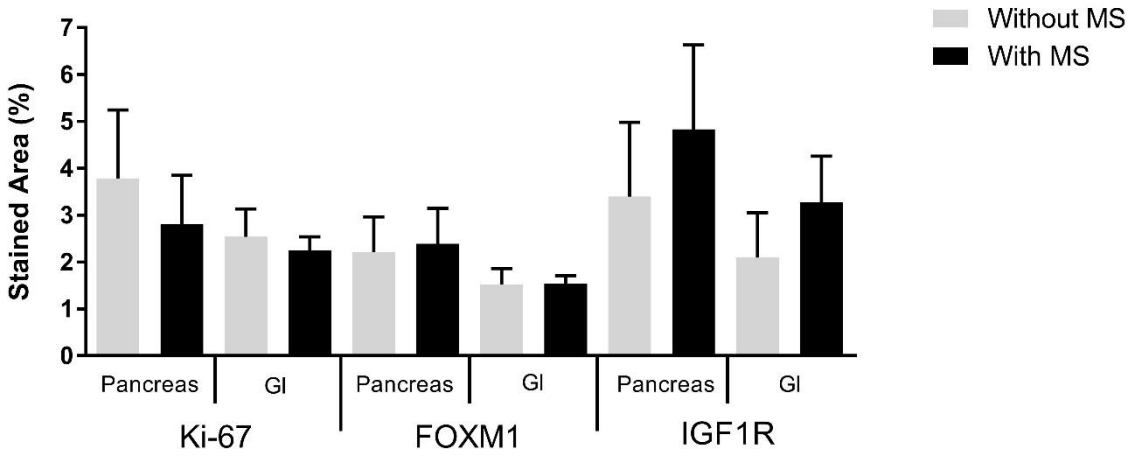
The immunohistochemical studies for appraisal of the expression of KI-67, FOXM1, IGF1R and IL-6, was performed in all tissue samples and the percentage of the stained area (tumor or peri-tumoral area) was calculated and determined using the ImageJ software (Figure 2).

Figure 2 - Histological photographs of ileal GI-NET and pancreatic NETs sections immunohistochemically stained for Ki-67, IGF1R, FOXM1 and IL-6.



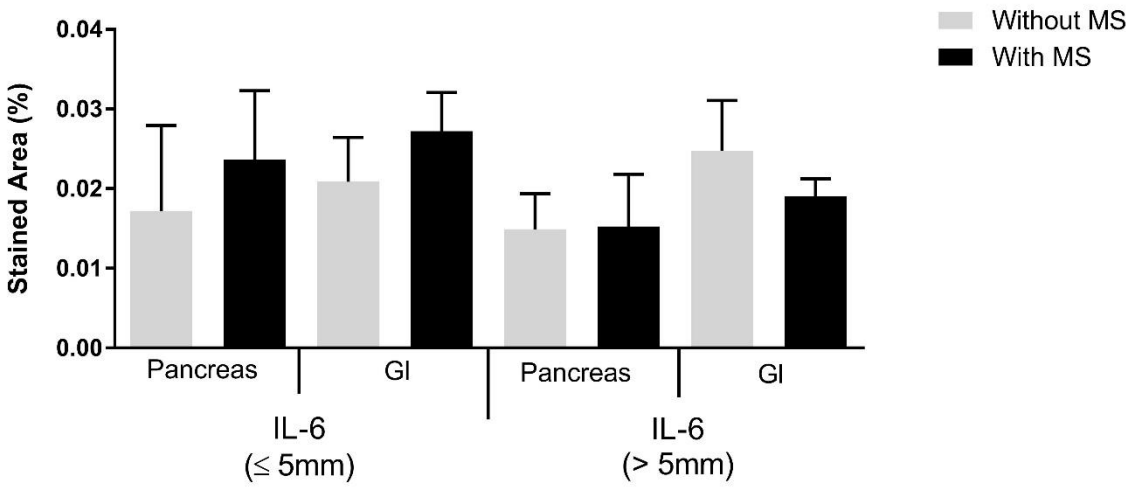
4.1- Expression of Ki-67, FOXM1, IGF1R and IL-6 markers in patients with or without metabolic syndrome

The percentages of the stained tumor areas in the patients with or without SM were not significantly different in both pancreatic and gastrointestinal NETs for all of the studied molecular markers, Ki-67, FOXM1 and IGF1R (Graph 1). Despite the absolute value of the percentage of stained area for IGF1R being higher in patients with MS both in pancreatic and gastrointestinal NETs it was not statistically significant (Graph 1).



Graph 1- Percentage of the stained area in GEP-NETs of patients with or without MS for the Ki-67, FOXM1 and IGF1R proteins.

The percentage of the IL-6 stained peri-tumoral area was assessed in two different distances from the tumor. The results showed that the IL-6 percentages of the stained areas were not statistically significantly different between patients with or without MS in pancreatic and gastrointestinal NETs, for both of the peri-tumoral areas at 5mm distance or less from the tumor limit and from higher than 5mm till the end of the tissue, (Graph 2). Regardless the absolute value of the percentage of stained area for IL-6, at less than 5 mm from the tumor limit in pancreatic and gastrointestinal NETs, were higher in patients with MS but not statistically significant (Graph 2).



Graph 2- Percentage of the stained peri-tumoral area of GEP-NETs of patients with or without MS at different distances for the IL-6 protein.

4.2- Expression of Ki-67, FOXM1, IGF1R and IL-6 markers in the patients with or without MS parameters

The percentages of the stained tumor areas for Ki-67, FOXM1 and IGF1R markers, were not significantly different between patients with or without each individual MS component both for pancreatic and gastrointestinal NETs (Table 7 and Table 8).

Table 7- Percentage of the tumor area stained in the immunohistochemical markers, Ki-67, FOXM1 and IGF1R, (mean±s.e.m.) in pancreatic NETs.

MS Components	Ki-67	FOXM1	IGF1R
BP normal:raised	2.74±1.28:3.73±1.27 p = 0.61	2.06±0.77:2.68±0.85 p = 0.62	3.18±1.67:5.51±1.98 p = 0.47
Fasting plasma glucose normal:raised	3.78±1.46:3.04±1.20 p = 0.91	2.67±0.67:2.28±0.89 p = 0.47	6.18±4.34:4.01±0.87 p = 0.51
Triglycerides normal:raised	4.06±1.30:2.85±1.24 p = 0.48	2.35±0.69:2.49±0.89 p = 0.76	2.88±1.10:6.22±2.30 p = 0.27
HDL normal:low	3.37±1.20:3.29±1.43 p = 0.84	2.02±0.61:2.85±1.01 p = 0.5	4.08±1.31:5.26±2.47 p = 0.71
Central obesity absent:present	3.02±1.76:2.55±0.73 p = 0.90	2.21±1.06:1.83±0.26 p = 0.64	3.90±2.60:4.98±2.08 p = 0.80

Table 8- Percentage of the tumor area stained in the immunohistochemical markers, Ki-67, FOXM1 and IGF1R, (mean±s.e.m.) in GI-NETs.

MS Components	Ki-67	FOXM1	IGF1R
BP normal:raised	2.42±0.57:2.30±0.30 p = 0.84	1.71±0.37:1.46±0.15 p = 0.77	2.83±1.15:2.94±0.96 p = 0.51
Fasting plasma glucose normal:raised	2.21±0.47:2.39±0.33 p = 0.99	1.38±0.32:1.61±0.17 p = 0.49	3.25±1.13:2.75±0.96 p = 0.77
Triglycerides normal:raised	2.48±0.48:2.16±0.38 p = 0.56	1.70±0.22:1.34±0.21 p = 0.23	3.70±1.25:1.93:0.56 p = 0.11
HDL normal:low	1.89±0.34:2.70±0.39 p = 0.13	1.27±0.18:1.76±0.22 p = 0.11	2.12±0.68:3.55±1.23 p = 0.28
Central obesity absent:present	2.39±0.42:2.30±0.36 p = 0.87	1.51±0.26:1.56±0.19 p = 0.86	1.93±0.72:3.36±1.15 p = 0.12

The percentage of the peri-tumoral area stained for the IL-6 marker assessed at two different distances from the tumor was not significantly different between patients with or without high fasting plasma glucose, high BP or raised triglycerides, both for pancreatic and gastrointestinal NETs (Table 9 and Table 10).

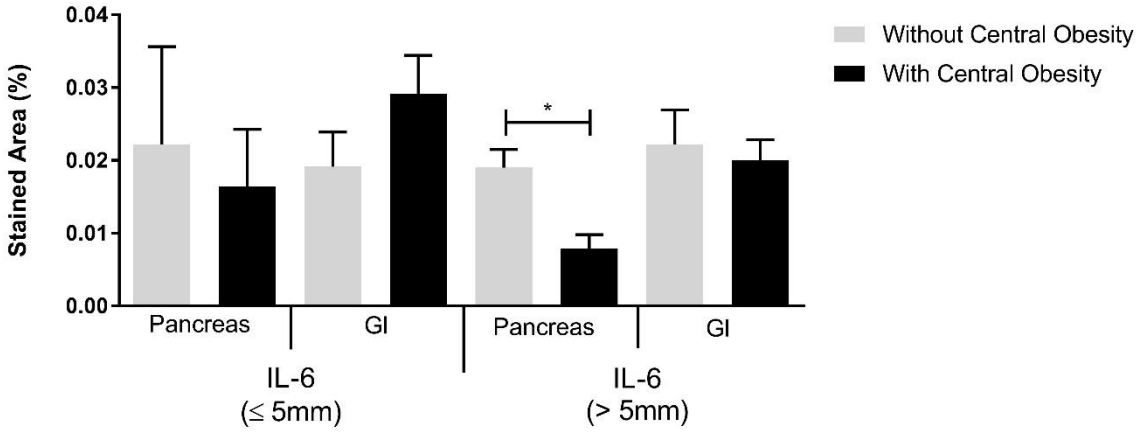
Table 9- Percentage of the peri-tumoral area stained by the immunohistochemical marker IL-6 (mean±s.e.m.) at two different distances from pancreatic NETs.

MS Components	IL-6 (≤5mm)	IL-6 (>5mm)
BP normal:raised	0.019±0.010**:**0.022±0.0091 p = 0.81	0.015±0.0036**:**0.014±0.0067 p = 0.35
Fasting plasma glucose normal:raised	0.019±0.010**:**0.022±0.0090 p= 0.91	0.014±0.0044**:**0.015±0.0065 p = 0.89
Triglycerides normal:raised	0.019±0.012**:**0.022±0.0081 p = 0.84	0.010±0.0047**:**0.018±0.0060 p = 0.35
HDL normal:low	0.024±0.011**:**0.017±0.0072 p = 0.84	0.013±0.0038**:**0.016±0.0077 p =0.69
Central obesity absent:present	0.022±0.013**:**0.016±0.0079 p = 0.71	**0.019±0.0025:0.0078±0.0019 p = 0.01**

Table 10 - Percentage of the peri-tumoral area stained by the immunohistochemical marker IL-6 (mean±s.e.m.) at two different distances from GI-NETs.

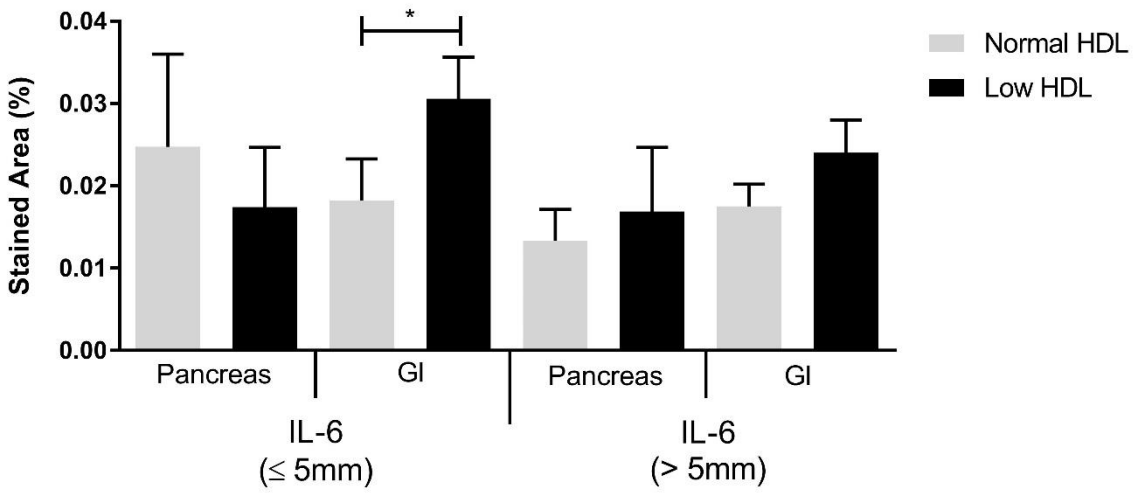
MS Components	IL-6 (≤5mm)	IL-6 (>5mm)
BP normal:raised	0.022±0.0055:0.026±0.0049 p = 0.68	0.025±0.0062:0.018±0.0022 p = 0.60
Fasting plasma glucose normal:raised	0.025±0.0053:0.024±0.0050 p = 0.47	0.023±0.0058:0.019±0.0022 p = 0.77
Triglycerides normal:raised	0.024±0.0050:0.026±0.0058 p = 0.75	0.020±0.0026:0.021±0.0044 p = 0.70
HDL normal:low	**0.018±0.0050:0.030±0.0050 p = 0.02**	0.017±0.0027:0.024±0.0040 p = 0.17
Central obesity absent:present	0.019±0.0047:0.029±0.0052 p = 0.13	0.022±0.0047:0.020±0.0028 p = 0.96

However, the percentage of the stained peri-tumoral area for IL-6 was statistically significantly higher within 5mm from the tumor limit till the end of the tissue in pancreatic NETs of patients without central obesity (0.019±0.0025) when compared with patients with central obesity (0.0078±0.0019) ($p<0.05$) (Table 9 and Graph 3).



Graph 3- Percentage of the stained peri-tumoral area of GEP-NETs in patients with or without central obesity at different distances for the IL-6 protein.

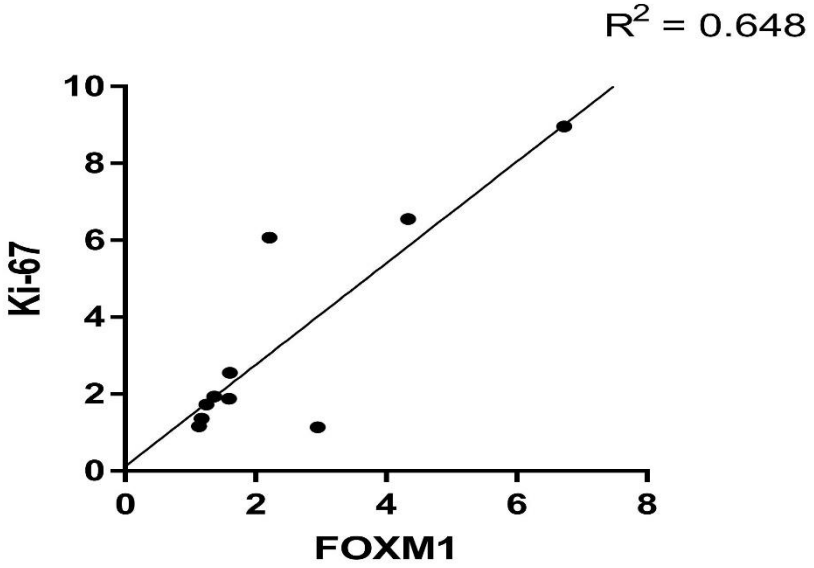
In the subset of patients with low HDL, IL-6 percentage of the stained peri-tumoral area at 5mm distance or less from the tumor limit in gastrointestinal NETs was significantly higher (0.030±0.0050) when compared with patients with normal HDL (0.018±0.0050) ($p<0.05$) (Table 10 and Graph 4).



Graph 4- Percentage of the stained peri-tumoral area of GEP-NETs in patients with or without low HDL at different distances for the IL-6 protein.

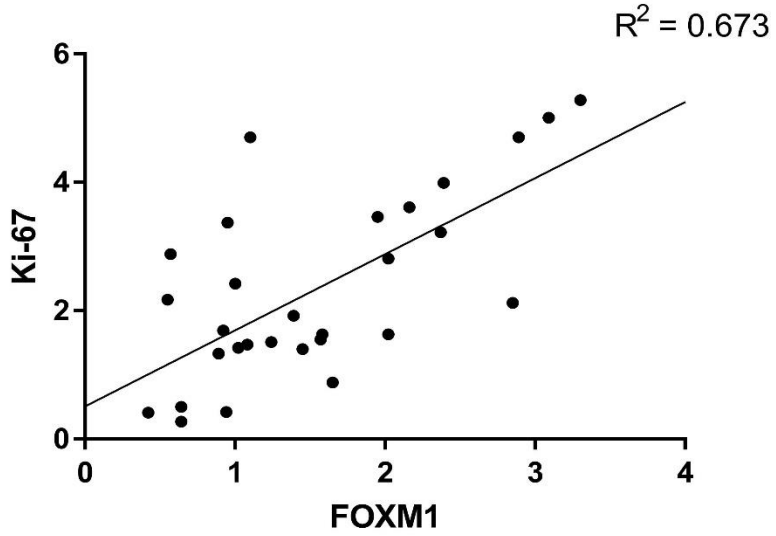
4.3- Correlations

For pancreatic NETs, a statistically significant positive correlation ($R^2 = 0.648$; $p< 0.043$) between the Ki-67 and FOXM1 was found (Graph 5). No other correlations between the studied proteins were found to be statistically significant in pancreatic NETs.



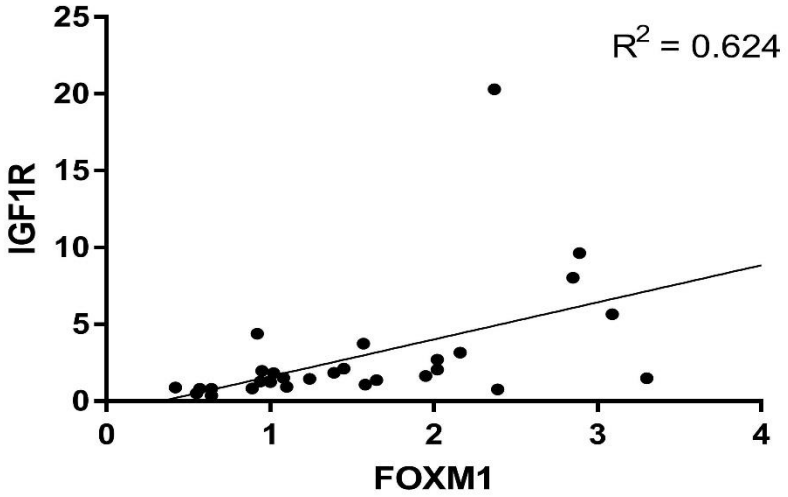
Graph 5 – Correlation between Ki-67 and FOXM1 proteins in pancreatic NETs.

For the gastrointestinal NETs, a statistically significantly positive correlation (R^2 = 0.673; p< 0.01) between Ki-67 and FOXM1 was found (Graph 6).



Graph 6- Correlation between Ki-67 and FOXM1 proteins in gastrointestinal NETs.

In addition, a statistically significantly positive correlation (R^2 = 0.624; p< 0.01) between IGF1R and FOXM1 was also found (Graph 7). No other correlations between the studied proteins were found to be statistically significant in gastrointestinal NETs.



Graph 7- Correlating between IGF1R and FOXM1 proteins in gastrointestinal NETs.

Chapter 5- Discussion

The link between MS criteria, its individual parameters and cancer has recently been established for different types of cancers. The association of MS with NETs and more specifically with GEP-NETs has not been so far reported, although deserves to be explored.

A possible link between insulin resistance, obesity, MS and GEP-NETs has been highlighted, from our previous case control study in which fasting plasma glucose was found to be significantly higher in patients harboring GEP-NETs when compared with controls, although neither MS criteria nor the reminder of its independent parameters were significantly different between the two groups (Santos et al., 2016). Furthermore, our subsequent studies between MS and GEP-NETs revealed more relevant associations between MS criteria, its individual parameters and GEP-NETs. These clinical results revealed that fasting plasma glucose, waist circumference, triglycerides and MS proportion were found to be significantly higher in patients with GEP-NETs when compared with controls whereas HDL was also significantly lower in patients with GEP-NETs (unpublished data). It was also found that elevated waist circumference, fasting plasma glucose, triglycerides and MS were risk factors for well-differentiated digestive NETs (unpublished data). Thus, the main aim of the research herein described was to gain further insight on the molecular links underlying the possible association between GEP-NETs and MS criteria and its individual parameters.

This study focused in evaluating how the expression of molecular markers that participate in metabolic and inflammatory pathways, in GEP-NETs and their surrounding tissue, correlates with the tumor clinical and pathologic features and presence of MS parameters.

To achieve this objective the selected molecules were Ki-67, FOXM1 and IGF1R as markers of the molecular pathways involved GEP-NETs biology and, IL-6 as inflammation marker in the peri-tumoral area.

The Ki-67 is a well-known protein that is present in all active phases of cell cycle and thus infer about the rate of cell growth/proliferation, therefore its expression is closely connected to cell proliferation and is routinely used as cell proliferation marker (Scholzen & Gerdes, 2000). In GEP-NETs, Ki-67 proliferation index is used to attribute the grade and classification of these tumors once their biological behavior is dependent on the degree of proliferation.

FOXM1 is an essential transcription factor that has been associated with a major role in cell proliferation, differentiation, cell cycle progression, tumorigenesis among others biological processes and its overexpression is essential in neoplastic cells of the majority of human

solid cancers, including GEP-NETs (Briest et al., 2015; Gomes, Zhao, & Lam, 2013; Halasi & Gartel, 2013).The overexpression of this crucial oncogenic transcription factor promotes resistance to chemotherapy and other genotoxic drugs of several human cancers leading to cancer cell growth and survival (Gomes, Zhao, & Lam, 2013; Halasi & Gartel, 2013; Halasi & Gartel, 2013). FOXM1 belongs to the PI3K-Akt-FOXO pathway, where it is an important downstream player of this cascade that can be repressed by wild type p53 or FOXO3a. This pathway is commonly deregulated in gastrointestinal NETs (Briest et al., 2015; Gomes, Zhao, & Lam, 2013; Halasi & Gartel, 2013; Wierstra, 2013). MAPK and PI3K pathways, the most frequently deregulated pathways in cancer, establish a crosstalk with FOXM1 that is also a transcriptional target of STAT3 (Halasi & Gartel, 2013; Wierstra, 2013).

IGF1 can be a major autocrine regulator of neuroendocrine tumor growth and secretion through the activation of complex molecular networks and IGF1R is one of the crucial TKRs in GEP-NETs biology (Briest & Grabowski, 2014; Raymond et al., 2011). Neuroendocrine cells that secrete a large amount of IGF-1, lead to IGF1R activation and consequently to a high expression of these TKRs and its ligand (Briest & Grabowski, 2014). IGF-1 and insulin are weak activators of MAPK pathway but strong activators of the PI3K-Akt-mTOR pathway (Raymond et al., 2011). So, IGF1 and IGF1R are highly activated in GEP-NETs and both IGF1 and IGF1 receptor are potential molecular targets for a variety of therapies in GEP-NETs. Nowadays, selective tyrosine kinase inhibitors and monoclonal antibodies are under clinical study and these results will help understand if IGF1R is or not a potential clinical target (Barbieri et al., 2014).

In the present study Ki-67, FOXM1 and IGF1R expression in pancreatic and gastrointestinal NETs was not significantly different between patients with or without MS or any of MS individual parameters (high BP, high fasting plasma glucose, high triglycerides, central obesity and low HDL). Notwithstanding the lack of epidemiological evidence disclosing the association between MS and GEP-NETs, the previously reported association between fasting plasma glucose and digestive neuroendocrine tumors and the increased risk for other cancers in patients with MS parameters it was expected that the expression of these markers could be altered in patients with at least some of the MS parameters in GEP-NETs (Esposito, Chiodini, Colao, Lenzi, & Giugliano, 2012; Lin, Ness-Jensen, Hveem, Lagergren, & Lu, 2015; Pothiwala, Jain, & Yaturu, 2009; “UMIB Summit 2015", 2016). However, our results showed that the presence of MS or any of its individual parameters do not significantly influence any of the studied markers in GEP-NETs. Nevertheless, a significant positive correlation between the Ki-67 and FOXM1 expression in pancreatic and gastrointestinal NETs was found. Knowing the important role of FOXM1 in cell cycle

progression and cell proliferation, this correlation suggest that FOXM1 and its pathway might be involved and responsible for cell proliferation in GEP-NETs. Moreover, this correlation was also previously reported in other study with gastrointestinal NETs (Briest et al., 2015). Therefore, inhibition of FOXM1 could be an important molecular target for GEP-NETs treatment, achievable either by a direct inhibition of FOXM1 protein or by an indirect inhibition of FOXM1 through the inhibition of some other player of the PI3K-Akt-FOXO pathway or other related in the PI3K, MAPK or STAT3 pathways (Briest et al., 2015; Gomes, Zhao, & Lam, 2013 Wierstra, 2013). Despite this and other previous study having demonstrated an increase of apoptosis *in vitro*, in a GEP-NETs cell line, after FOXM1 inhibition, further studies are needed before its applicability in the routine clinical practice (Bhat, Zipfel, Tyler, & Gartel, 2008; Briest et al., 2015). In addition, FOXM1 and IGF1R expression were also found to be positively correlated in gastrointestinal NETs. This correlation suggests that FOXM1 expression could be stimulated and activated by IGF1R activity or vice-versa in gastrointestinal NETs and thereby this could be an additional treatment target. Besides that, IGF1 are strong activators of the PI3K-Akt-mTOR pathway and a study in cardiomyocytes showed that a downregulated FOXM1 expression is associated with a downregulated IGF1R expression (Briest & Grabowski, 2014; Sengupta, Kalinichenko, & Yutzey, 2012). In NETs, IGF1 and IGF1R are highly expressed and probably can lead to the expression of FOXM1, likely due to the activation of the PI3K-Akt-FOXO pathway (Briest & Grabowski, 2014; Furukawa et al., 2005).

Il-6 is a pro-inflammatory cytokine involved mainly in a chronic inflammatory environment (Briest & Grabowski, 2014). IL-6 is responsible for shaping the tumor microenvironment as one of the most expressed cytokines in the tumor surrounding tissues (Briest & Grabowski, 2014). Usually, IL-6 tips the balance into pro-inflammatory activity and thus initiation of chronic inflammation, resulting in a support to tumor growth and progression via the stimulation of different pathways and several downstream effectors (Briest & Grabowski, 2014).

Although there is not a clear definition for peri-tumor area, as previous studies have used a wide range of distances from the tumor limit as adjacent tumor tissue, spanning from a few millimeters to 2 cm wide (Balsat et al., 2013; Zhuang et al., 2013). We have selected the maximum distance of 5mm from the tumor limits, whenever available, as definition of peri-tumoral tissue to study the IL-6 expression and its variation within this and outside of this distance to evaluate the inflammatory tonus in the tumor microenvironment, based in previous studies for other markers described in the literature (Balsat et al., 2013; Zhuang et al., 2013).

IL-6 expression in the peri-tumoral tissue was not found to be significantly different between patients with and without MS in GEP-NETs, both within the 5mm or less from the tumor limit and 5mm or higher till the tissue limit. Admitting that MS is associated with a chronic low grade inflammatory state, IL-6 expression was expected to be higher in GEP-NETs patients with MS, however our results do not favor that IL-6 exerts an influence in GEP-NETs pro-inflammatory microenvironment. It was expected for IL-6 expression in the peri-tumoral to be higher in patients with at least one of MS individual components, as each one of these parameters have been individually linked with inflammation. In fact, high blood pressure could be a stimulus for inflammation, high triglycerides levels increase inflammation by the activation of Nf-KB pathway and insulin resistance is associated with a chronic low grade inflammatory state (Chae, Lee, Rifai, & Ridker, 2001; Welty, 2013; Zeyda & Stulnig, 2009). Despite this, IL-6 expression was not significantly different between patients with or without high fasting plasma glucose, high BP or raised triglycerides, both for pancreatic and gastrointestinal NETs. Thus, suggesting that none of these parameters contributes to the inflammation in the tumor microenvironment or that it does not influences IL-6 expression. Furthermore, IL-6 expression at the peri-tumoral area in pancreatic NETs was lower in patients with central obesity, which is contradictory with what was previously described in the literature once obesity is consistently associated with a chronic low grade inflammatory state (Zeyda & Stulnig, 2009). Moreover, IL-6 expression in the peri-tumoral area of gastrointestinal NETs was significantly higher in patients with low HDL when compared with samples of patients with normal HDL, suggesting that low HDL in gastrointestinal NETs contributes to an inflammatory environment in the peri-tumoral area, unsurprisingly as HDL anti-inflammatory properties have been extensively described before (Welty, 2013).

In short, knowing the important role of FOXM1 in cell cycle progression and cell proliferation FOXM1 could be an important molecular target for GEP-NETs therapy, due to a positive correlation with Ki-67, and in gastrointestinal NETs, due to a positive correlation with IGF1R, FOXM1 activity might be activated by the expression of IGF1R, likely due to the activation of the PI3K-Akt-FOXO pathway.

Chapter 6- Conclusion

The present study has shown that Ki-67, FOXM1 and IGF1R expression in pancreatic and gastrointestinal NETs was not significantly different between patients with or without MS or any of MS individual parameters (high BP, high fasting plasma glucose, high triglycerides, central obesity and low HDL). It was observed a significant positive correlation between the Ki-67 and FOXM1 expression in pancreatic and gastrointestinal NETs. It was also observed a significant positive correlation between the FOXM1 and IGF1R expression in gastrointestinal NETs. IL-6 expression in the peri-tumoral tissue was not found to be significantly different between patients with or without MS or some MS individual parameters (high fasting plasma glucose, high BP or raised triglycerides) in GEP-NETs, both within the 5mm or less from the tumor limit and 5mm or higher till the tissue limit. On the other hand, IL-6 expression at the peri-tumoral area in pancreatic NETs was lower in patients with central obesity and IL-6 expression in the peri-tumoral area of gastrointestinal NETs was significantly higher in patients with low HDL when compared with samples of patients with normal HDL.

These results suggest that the presence of MS or any of its individual parameters do not significantly influence any of the studied markers and thus related pathways in GEP-NETs, except for IL-6 in two of its individual parameters. For IL-6 expression at the peri-tumoral area of gastrointestinal NETs the results suggest that low HDL in gastrointestinal NETs contributes to an inflammatory environment in the peri-tumoral area. Contrariwise, for IL-6 expression at the peri-tumoral area in pancreatic NETs the results suggest a lower expression with central obesity, which is contradictory with what was previously described in the literature. At last but not least, the results suggest that FOXM1 and its pathway might be involved and responsible for cell proliferation in GEP-NETs and thus inhibition of FOXM1 could be an important molecular target for GEP-NETs treatment. Furthermore, the results suggest that FOXM1 expression are related to IGF1R in gastrointestinal NETs and thereby this could be an additional treatment target.

Futures studies will be required in order to understand the link and influence of MS criteria, its individual parameters with NETs, more specifically with GEP-NETS. For this, some markers and pathways associated with GEP-NETs biology, such as vascular endothelial growth factor, C-X-C chemokine receptor type 4, epidermal growth factor, players involved in PI3K and MAPK pathways, Nf-KB pathway and other cytokines, could be studied. Moreover, studies will also be required to obtain a proper understanding of FOXM1 protein/pathway role and the consequences of its inhibition in GEP-NETs in order to assess its applicability in the routine clinical practice. Finally, a study of IGF1R and FOXM1

interaction is needed in gastrointestinal NETs to understand the potential of this possible additional treatment target.

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